Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice

J. Justin Milner, Jenny Rebeles, Suraj Dhungana, Delisha A. Stewart, Susan C. J. Sumner, Matthew H. Meyers, Peter Mancuso and Melinda A. Beck

J Immunol 2015; 194:4846-4859; Prepublished online 10 April 2015; doi: 10.4049/jimmunol.1402295
http://www.jimmunol.org/content/194/10/4846

Supplementary Material http://www.jimmunol.org/content/suppl/2015/04/10/jimmunol.1402295.DCSupplemental

References This article cites 60 articles, 29 of which you can access for free at: http://www.jimmunol.org/content/194/10/4846.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Obesity Increases Mortality and Modulates the Lung Metabolome during Pandemic H1N1 Influenza Virus Infection in Mice

J. Justin Milner,* Jenny Rebello,* Suraj Dhungana,† Delisha A. Stewart,† Susan C. J. Sumner,‡ Matthew H. Meyers,* Peter Mancuso,‡ and Melinda A. Beck*

Obese individuals are at greater risk for hospitalization and death from infection with the 2009 pandemic H1N1 influenza virus (pH1N1). In this study, diet-induced and genetic-induced obese mouse models were used to uncover potential mechanisms by which obesity increases pH1N1 severity. High-fat diet–induced and genetic-induced obese mice exhibited greater pH1N1 mortality, lung inflammatory responses, and excess lung damage despite similar levels of viral burden compared with lean control mice. Furthermore, obese mice had fewer bronchoalveolar macrophages and regulatory T cells during infection. Obesity is inherently a metabolic disease, and metabolic profiling has found widespread usage in metabolic and infectious disease models for identifying biomarkers and enhancing understanding of complex mechanisms of disease. To further characterize the consequences of obesity on pH1N1 infection responses, we performed global liquid chromatography–mass spectrometry metabolic profiling of lung tissue and urine. A number of metabolites were perturbed by obesity both prior to and during infection. Uncovered metabolic signatures were used to identify changes in metabolic pathways that were differentially altered in the lungs of obese mice such as fatty acid, phospholipid, and nucleotide metabolism. Taken together, obesity induces distinct alterations in the lung metabolome, perhaps contributing to aberrant pH1N1 immune responses. The Journal of Immunology, 2015, 194: 4846–4859.

The triple reassortant H1N1 influenza virus (pH1N1) caused the first pandemic of the 21st century in 2009, and this strain continues to circulate and contribute to seasonal influenza epidemics globally (1, 2). Although infection with the pH1N1 strain typically results in relatively mild, uncomplicated symptoms, a number of epidemiological investigations have identified obesity as an independent risk factor for hospitalization and death to pH1N1 (3–6). More than 500 million individuals are obese (body mass index $\geq$ 30 kg/m²) globally (7), and thus understanding the mechanisms by which excess adiposity drives greater pH1N1 infection severity is critical for solving this public health threat. Similar to humans, obese mice are also more susceptible to influenza infection mortality compared with lean controls (8, 9). Several reports have demonstrated that obesity alters inflammatory and pathological responses in the lung during influenza infection in mice, but the underlying mechanisms causing these aberrant immune responses and ultimately death remain unclear (10–15). Excess accumulation of adipose tissue triggers metabolic and physiologic perturbations such as insulin resistance, hyperleptinemia, oxidative stress, low-grade chronic inflammation, and alterations in a variety of circulating nutrients and hormones, all of which could potentially affect influenza immunity and disease severity (8, 16).

Although our understanding of host immune responses to influenza virus infection in vitro and in vivo are well established, much remains unknown regarding the mechanisms in which perturbations in systemic metabolism may impact influenza immune responses and infection mortality. This is pertinent because not only is obesity a highly prevalent metabolic disease, but other risk factors for severe influenza infections, such as heart disease, diabetes, pregnancy, and aging (17, 18), are also associated with distinct cellular and systemic metabolic complications (16, 19, 20). Metabolic profiling has been useful for identifying biomarkers or uncovering complex mechanisms in a number of metabolic diseases such as cardiovascular disease, type 2 diabetes, and obesity (21, 22). Furthermore, application of this methodology to infectious diseases models continues to gain momentum, facilitating greater understanding of the complex interactions between pathogen and host and identifying prognostic or diagnostic biomarkers/metabolic signatures unique to certain disease states and stages (23–26). Although lipidomics has recently proven useful in identifying lipid metabolites that have antiviral effects (27) or serve as influenza biomarkers (28), metabolomics has only been applied to a few influenza models in vitro (29, 30) or in vivo (26, 31). Relatively little is known regarding the consequences of influenza virus infection on
the global lung metabolome (at the site of infection) or how altered systemic metabolism (e.g., obesity) may impact influenza pathogenesis and metabolic processes in the lung.

In this study we used two models of obesity, diet- and genetic-induced, providing a robust characterization of the immunological and metabolic consequences of obesity during H1N1 infection. High-fat diet (HFD)-induced and genetic-induced obese mice exhibited greater H1N1 mortality, as well as elevated lung inflammatory responses and excess lung damage, despite similar viral titers compared with lean control mice. Additionally, both models of obesity exhibited distinct alterations in immune cell populations, such as fewer macrophages and regulatory T cells (Tregs) in the airways. We also demonstrated that the lung metabolome was differentially altered by obesity during influenza virus infection. Furthermore, ultra-performance liquid chromatography–mass spectrometry (UPLC-MS) profiling successfully distinguished urine samples from infected lean and obese mice as early as 2 d postinfection (dpi), and the urine from infected obese mice reflected alterations in a diverse number of metabolic pathways. Pathway enrichment analyses, based on the uncovered metabolic signatures in lung tissue and urine, revealed differentially regulated metabolic processes that perhaps may be contributing to greater H1N1 severity in obese mice, such as fatty acid, phospholipid, and nucleotide metabolism. Taken together, the present study provides an in-depth analysis of the immunological and metabolic consequences of obesity during influenza virus infection.

Materials and Methods

Mice and diets

Diet-induced obesity was achieved by maintaining weanling, male C57BL/6J mice (obtained from The Jackson Laboratory, Bar Harbor, ME) on a HFD (60% kcal fat, Research Diets, New Brunswick, NJ, D12492 formula), and lean mice were maintained on a low-fat diet (LFD; 10% kcal fat, Research Diets, D12450B formula) or a standard chow diet (CD; 14% kcal fat, Purina RMH 3000, LabDiet, St. Louis, MO) for 4–16 wk. The HFD and LFD were nutritionally defined and contain purified ingredients. The HFD and LFD are nutritionally matched except for fat content and carbohydrate content (70% kcal in LFD and 20% kcal in HFD provided by carbohydrates). For the CD, calories are derived from 26% protein, 14% fat, and 60% carbohydrate.

Genetically induced obesity was achieved by crossing fully floxed leptin receptor mice on a C57BL6/J background (provided by Dr. Alyssa Hasty) with C57BL/6J-Tg(Nkx2.1-cre)22SandJ mice purchased from The Jackson Laboratory. Fully floxed mice expressing the Cre transgene under control of the Nkx2.1 promoter (LepRH−/−) lack leptin receptor signaling in hypothalamic neurons (LepRβ−/−) and become obese owing to hyperphagia (32). Heterozygous breeding using LepRH+/− mice was necessary because homozygous LepRH−/− mice do not produce offspring (32). Mice were genotyped as previously described (32) and housed in isolation cubicles at the University of North Carolina, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

All procedures involving the use of mice were fully approved by the University of North Carolina Institutional Animal Care and Use Committee.

Influenza virus infection and viral titers

Influenza A/California/04/2009 (BEI Resources, Bethesda, MD) was propagated in embryonated hen’s eggs and titrated via a modified TCID50 in replicates of four.

BALF albumin was measured with a mouse albumin ELISA kit (GenWay Biotech, San Diego, CA). Total protein in BALF was measured via a standard BCA assay (BCA kit, Sigma-Aldrich, St Louis, MO). BALF cytokines (IL-4, IFN-γ, MCP-1, RANTES, KC, IL-17A, IL-10, and TNF-α) were measured using a Bio-Plex assay (Bio-Rad, Hercules, CA) per the manufacturer’s instructions. IL-4 was not measurable above the lower limit of the assay.

Lung histopathology

The left lobe of the lung was inflated and fixed with 10% neutral buffered formalin. Fixed lung tissue was embedded in paraffin and processed for H&E staining by the University of North Carolina Animal Histopathology Core Facility. The extent of lung immune cell infiltration was scored blindly according to a scale from 0 to 4: 0, no inflammation; 1, mild influx of inflammatory cells; 2, increased inflammation with 25–50% of the total lung involved; 3, severe inflammation involving 50–75% of the lung; and 4, almost all lung tissue contains inflammatory infiltrate (12, 14).

Flow cytometry

Lung, mediastinal lymph nodes (mLN), and BAL cells were stained for flow cytometry as previously described (14). For staining of BAL cells from uninfected mice, BAL samples were pooled from two individual mice to obtain a sufficient number of cells (two pooled BAL samples represent n = 1).

Urine samples (25 µl) were diluted with acetonitrile containing tryptophan-d5 internal standard and were homogenized using a bead beater operating at 1750 rpm. A 300-µl sample of lung homogenate was spiked with tryptophan-d5 internal standard, lyophilized, reconstituted in 100 µl 95:5 water/methanol, and 10 µl was injected into a SYNAPT G2 quadrupole time-of-flight mass spectrometer coupled to an Acuity UPLC (Waters, Milford, MA) for broad-spectrum metabolomics analysis. The lung metabolites were separated on a Waters Acquity BEH T3 column (2.1 × 100 mm, 1.8 µm particle size) operating at 50°C using a reversed-phase chromatographic method. A gradient mobile phase consisting of water, formic acid, acetonitrile, and methanol was used for sample preparation.

SALF and serum leptin levels were measured using the commercial ELISA kits. Total protein in BALF was measured with a moose albumin ELISA kit (GenWay Biotech, San Diego, CA). Total protein in BALF was measured via a standard BCA assay (BCA kit, Sigma-Aldrich, St Louis, MO). BALF cytokines (IL-4, IFN-γ, MCP-1, RANTES, KC, IL-17A, IL-10, and TNF-α) were measured using a Bio-Plex assay (Bio-Rad, Hercules, CA) per the manufacturer’s instructions. IL-4 was not measurable above the lower limit of the assay.

Influenza virus infection and viral titers

Influenza A/California/04/2009 (BEI Resources, Bethesda, MD) was propagated in embryonated hen’s eggs and titrated via a modified TCID50 using hemagglutination as an endpoint and evaluated by the method of Reed and Muench (33) as previously described (8, 12). Mice were lightly anesthetized via isoflurane inhalation and were infected intranasally with 0.05 ml 5.8 TCID50 or 1.3 TCID50 as described in the figure legends. For diet-induced obese mouse models, mice were infected at 17–19 wk of age. LepRHfl/fl and LepRHfl/fl mice were infected at 12–25 wk of age (LepRHfl/fl and LepRHfl/fl were age matched).

For influenza virus infection and viral titers of infected mice, bronchoalveolar lavage fluid (BALF) was titrated via a modified TCID50 in replicates of four.
Quantification of lung gene expression

Total RNA was isolated from lung tissue via the TRizol method (Invitrogen), and reverse transcription was performed a SuperScript II first-strand synthesis kit (Invitrogen) and oligo(dT) primers. Cytokine and chemokine expression were quantified using quantitative RT-PCR as previously described (36). Gene expression levels were normalized to β-actin.

Statistical analysis

For all statistical analyses, JMP statistical software (SAS Institute, Cary, NC) or GraphPad (San Diego, CA) were used. For parametric data (including metabolites), statistical significance was evaluated using a two-way ANOVA or a two-tailed unpaired Student t test. Nonparametric data were evaluated using the Wilcoxon signed rank test or Kruskal–Wallis test. Mean values were considered statistically significant at p ≤ 0.05. The log-rank test was used to compare percentage survival.

Results

Diet-induced obese mice are more susceptible to pH1N1 mortality compared with CD-fed and purified LFD-fed lean mice

Epidemiological evidence demonstrates that obesity increases the likelihood of severe influenza infection complications in humans (4, 6, 17, 37, 38), and several mouse models have also confirmed this outcome (10–15, 36). Obesity exacerbates lung inflammation and pathology, alters lung immune cell populations, impairs lung healing, and in some cases increases viral titers during influenza infection in mice (10–15, 36). To further investigate the consequences of obesity on pH1N1 immunity, weanling male C57BL6/J mice were fed a purified HFD (60% kcal fat), a purified LFD (10% kcal fat, nutritionally matched to the HFD except for fat and carbohydrate content), or a standard CD (14% kcal fat). The two low-fat control diets (LFD and CD) were included because although previous studies have used both of these diets as controls for a HFD, they have never been directly compared with each other in the same study (10–15, 36). As expected, mice fed a HFD gained significantly more weight compared with LFD- and CD-fed mice (Fig. 1A). Although LFD and CD mice exhibited relatively similar levels of weight gain in comparison with HFD-induced obese mice, LFD mice weighed significantly more than CD mice after 8 wk on the diet. Obesity results in metabolic perturbations, reflected by alterations in a number of circulating hormones and nutrients (16). In Fig. 1B, we demonstrate that HFD-fed obese mice had elevated fasting blood glucose measures compared with LFD and CD mice. Furthermore, serum insulin was elevated in HFD mice compared with CD mice, and LFD mice exhibited a relatively intermediate concentration. Additionally, HFD mice had a greater concentration of serum TAG and leptin but lower adiponectin levels. In summary, HFD mice exhibited prototypical characteristics of obesity, whereas LFD mice exhibited a somewhat intermediate phenotype compared with HFD and CD mice.

After 14–16 wk on the diet, mice were infected with 5.8 \times 10^2 TCID_{50} of the 2009 pandemic H1N1 virus, influenza A/Cal/04/09 (pH1N1). Strikingly, although no CD mice succumbed to the pH1N1 infection, 40% of LFD mice and greater than 80% of HFD mice died by 10 dpi (Fig. 1C). HFD-fed obese mice exhibited a significantly higher mortality rate compared with both LFD and CD mice. Although it has been shown that diet-induced obese mice are more susceptible to pH1N1 mortality (10), it has never been demonstrated that LFD-fed mice are also more likely to die of influenza infection compared with CD mice. Analysis of absolute weight loss and percentage weight loss demonstrated that CD mice recover faster from the infection compared with HFD and LFD mice (Fig. 1D, 1E).

Given that all CD mice survived the infection, we then tested whether the discrepancy in pH1N1 mortality between the three dietary groups was maintained with a greater pH1N1 dose. All three dietary groups were infected with 1.3 \times 10^7 TCID_{50}, and by 7 dpi, 83% of HFD mice died (Fig. 1F) compared with 55% of LFD mice and only 33% of CD mice. Despite the increased death in all dietary groups compared with the previous lower dose, the mortality differences between the dietary groups were relatively maintained in that HFD and LFD mice were more susceptible to mortality compared with CD mice (p < 0.005 and p = 0.05, respectively), and HFD mice trended toward increased mortality compared with LFD mice (p = 0.08). There were no differences in total weight loss between the three groups (Fig. 1G), but HFD mice displayed a significantly lower percentage weight loss during the course of the infection compared with LFD and CD mice (Fig. 1H), which has been reported previously (10). In summary, we show that HFD-induced obesity increases pH1N1 infection severity compared with two different lean mouse models. Given that LFD mice exhibited greater mortality than did CD mice, simply changing the components of a diet (without inducing any nutritional deficiencies) can modulate influenza infection severity, and thus we cannot rule out synergistic effects of a HFD and obesity.

Diet-induced obese mice exhibit greater lung damage and inflammation during a pH1N1 infection

To assess potential mechanisms for the discrepancies in pH1N1 mortality between dietary groups, we measured the viral titers in the lung airways of CD, LFD, and HFD mice infected with 5.8 \times 10^2 TCID_{50}. There were no differences in viral titers among the three groups at 4 or 8 dpi (Fig. 2A). Although some studies report that obesity does not impact influenza titers (10, 36), it has also been shown that obesity can increase pH1N1 titers (11).

We then assessed the level of cellular infiltration and lung pathology during the pH1N1 infection. HFD, LFD, and CD mice exhibited similar BAL and lung total cell numbers during the infection (Fig. 2B, 2C). As expected, H&E-stained lungs revealed that pH1N1 infection caused distinct pathological changes in the bronchioles, vessels, and alveoli of all three dietary groups (Fig. 2D). At 4 dpi, partial bronchiole denuding and perivascular cuffing occurred in all three dietary groups. By 8 dpi, perivascular cuffing, bronchial denuding, and immune cell infiltration increased in all three dietary groups compared with the day 4 time point. Histopathology scores of total lung infiltration revealed there were no significant differences among the three dietary groups (data not shown), consistent with similar total BAL and lung cell numbers.

To assess the level of damage to the alveolar/endothelial barrier, total protein and albumin were measured in BALF from pH1N1-infected CD, LFD, and HFD mice (Fig. 2E, 2F). At 4 dpi, HFD mice exhibited a greater fold increase in BALF protein compared with LFD and CD mice (Fig. 2E). Furthermore, at 8 dpi, HFD mice displayed a greater fold increase in BALF protein compared with CD mice. Additionally, HFD mice exhibited a greater fold increase in BALF albumin compared with LFD and CD mice at 4 dpi (Fig. 2F). Therefore, obesity enhances lung epithelial cell injury during pH1N1 infection despite no differences in viral burden or total leucocyte recruitment.

Cytokine and chemokine production in the lung airways of the three dietary groups was also measured via a multiplex assay to assess inflammatory responses (Fig. 2G). HFD mice had elevated levels of the keratinocyte chemoattractant (KC or CXCL1) and MCP-1 compared with LFD and CD mice at 8 dpi. TNF-α was greater in HFD and CD mice at 8 dpi compared with LFD mice. There were no differences in IFN-γ, IL-10, IL-17A, or RANTES. Taken together, despite no differences in lung inflammatory cell infiltration and viral titers, HFD mice exhibited greater lung injury and production of inflammatory cytokines during pH1N1 infection.
HFD-induced obese mice have fewer macrophages and Tregs in the lung airways compared with lean mice

To gain a better understanding of the causes and consequences of greater lung injury and alterations in chemokine expression in HFD mice, the distribution of innate immune cells in the airways of pH1N1-infected CD, LFD, and HFD mice was assessed. Fig. 3A is a representative flow cytometry histogram for identification of macrophages, neutrophils, and NK cells. Fig. 3B represents the percentages (top) and numbers (bottom) of F4/80+ macrophages, NK1.1+ NK cells, and Ly6G+ neutrophils. Unexpectedly, HFD mice had significantly fewer BAL macrophages at 4 dpi, trending fewer NK cell numbers (\( p = 0.07 \)) as well as similar numbers of neutrophils with CD mice. Although HFD mice had greater levels of KC and MCP-1 expression (neutrophil and macrophage chemokines, respectively), this did not result in greater infiltration of macrophages or neutrophils.

We then measured the distribution of CD4+ and CD8+ T cells in the BAL, lung, and mLN of the three dietary groups. HFD mice had fewer BAL CD4+ T cells at 4 dpi with no differences in CD8+ T cell numbers compared with CD mice (Fig. 3C, 3D). Given the alterations in T cell numbers detected in the lung airways, CD4+ and CD8+ T cell numbers were measured in lung tissue and in mLN at 4 and 8 dpi. There were no differences in lung or mLN T cell numbers between CD and HFD mice (data not shown).

CD4+Foxp3+ Tregs have been shown to curtail inflammatory responses to respiratory syncytial virus and some influenza infection models in mice (39–41). Because we detected fewer BAL CD4+ T cells in HFD mice, we also suspected fewer BAL Tregs as well, perhaps contributing to heightened lung inflammation and damage in HFD mice. Fig. 3E is a representative gating scheme for identification of BAL Tregs in CD, LFD, and HFD mice. HFD mice had a significantly greater percentage of Tregs at 4 dpi (Fig. 3E), and consistent with fewer CD4+ T cells at 8 dpi, HFD mice had fewer Tregs at 8 dpi (Fig. 3F). Additionally, the level of Treg activation via CD103 expression was measured (42), and HFD mice had fewer CD103+ BAL Tregs as well (Fig. 3G). Given the discrepancy in Treg number in the lung airways, Treg distribution in lung tissue and mLN were also assessed (Fig. 3H, 3I). HFD mice had fewer Tregs at 0 and 4 dpi in the lung tissue and fewer at 8 dpi in the mLN compared with LFD mice.

Metabolic profiling distinguishes lung samples from uninfected and infected obese mice

Obesity is inherently a metabolic disease, and metabolic profiling has found widespread usage in infectious disease models for identifying biomarkers and improving our understanding of these complex diseases (23–26). Therefore, we hypothesized that metabolic profiling would not only provide a snapshot of the metabolic consequences of obesity in the lung during pH1N1 infection, but it would also shed...
light on potential mechanisms driving greater pH1N1 severity in obese mice. Lungs were harvested from CD, LFD, and HFD mice at 0, 4, and 8 dpi, and UPLC-MS metabolic profiling was performed. In Fig. 4A, the OPLS-DA plot shows significant separation between lung samples from uninfected CD and HFD mice. As one might expect, several metabolites were altered by the HFD and/or obesity, and most of these metabolites were related to lipid metabolism (involved in fatty, cholesterol, or phospholipid metabolic pathways). Once infected, the HFD and CD lung metabolic profiles continued to separate (Fig. 4B). An increase in differential metabolites was observed in HFD infected mice at 4 dpi, particularly in phospholipid and nucleotide metabolites (Fig. 4B).

By 8 dpi, the number of metabolites significantly altered by obesity more than doubled compared with uninfected lung tissue (Fig. 4C). Fatty acid– and phospholipid-related species comprised most significantly altered metabolites in HFD mice at 8 dpi. Furthermore, several amino acid and ketone metabolites were detected at high levels in the lungs [with (S)-3-hydroxybutyric acid detected at a 17.5-fold greater concentration in lungs of HFD mice, \( p < 0.0005 \)]. Few of the differentially altered metabolites in uninfected HFD mice were also different at 8 dpi, indicating that the detected metabolic changes occurring in the lung during infection are not simply due to obesity or HFD feeding, but are a consequence of the infection in the HFD-induced obese mice.

Lastly, we determined whether the unique metabolic changes that were measured in the lungs of HFD mice at 8 dpi may provide a snapshot of some of the altered dynamic metabolic processes occurring during the infection. We used the GeneGo MetaCore systems biology software to uncover differentially altered metabolic pathways (Fig. 4D). The most significantly altered pathway in the lungs of obese mice at 8 dpi was N-acyltransferase metabolism (\( p < 0.0005 \)), as well as a number of fatty acid–related pathways and nucleotide metabolism pathways (Fig. 4D). For metabolite comparisons, we primarily focused on differences between
CD and HFD mice because we detected the greatest discrepancy in infection severity between these two groups. However, a complete list of differentially altered metabolites between CD, LFD, and HFD mice is well as fold change and p values are listed in Supplemental Table I.

**Obesity, independent of diet, increases pH1N1 mortality**

HFD mice exhibited greater pH1N1 infection severity, alterations in inflammatory immune responses, and diverse changes in lung metabolism during the infection. However, a limitation of utilizing a diet model of obesity is that differential outcomes between lean (CD or LFD) and HFD obese mice could be influenced by differences in the diets, potentially confounding the effects of obesity. Furthermore, LFD mice display greater mortality compared with CD mice, suggesting that simply modulating the diet (without causing any nutritional deficiencies) can impact pH1N1 infection outcome, although not to the same extent as in HFD obese mice. Therefore, we included a genetic model of obesity to...
assess the impact of obesity on infection responses independent of dietary effects.

We used a genetic model of obesity in which excess adiposity is driven primarily by hyperphagia (all mice are fed a CD). It has previously been shown that \(\text{ob/ob}\) mice, a robust model of genetic obesity, are more susceptible to pH1N1 infection compared with wild-type mice (10). However, utilization of the \(\text{ob/ob}\) mouse model has a number of limitations due to the global deficiency of leptin signaling. Leptin is critical for proper physiology and immunity, and leptin deficiency has been shown to impair host defenses, thus confounding the immunomodulatory consequences of obesity (8). Therefore, we used a tissue-specific model, previously characterized by Ring and Zeltser (32), in which disruption of leptin signaling is limited to hypothalamic neurons (the primary site of leptin-mediated appetite control) to further address the impact of obesity on pH1N1 immunity. These mice become rapidly obese and exhibit hyperinsulinemia, hyperglycemia, and hyperleptinemia among other metabolic alterations characteristic of obesity (32).

Male (Fig. 5A) and female (Fig. 5B) LepRH\(^{2/2}\) mice, lacking functional leptin signaling in hypothalamic neurons, rapidly gained excess body weight compared with lean LepRH\(^{fl/fl}\) and LepRH\(^{+/2}\) mice. At 13–16 wk of age, LepRH\(^{2/2}\), LepRH\(^{+/2}\), and LepRH\(^{fl/fl}\) mice were infected with \(5.8 \times 10^2\) TCID\(_{50}\) pH1N1. Strikingly, obese male (Fig. 5C) and female LepRH\(^{2/2}\) (Fig. 5D) mice were significantly more susceptible to pH1N1 mortality compared with lean LepRH\(^{+/2}\) and LepRH\(^{fl/fl}\) mice. Obese male and female LepRH\(^{2/2}\) mice lost more absolute weight during the pH1N1 infection compared with both lean groups (Fig. 5E, 5F).

**FIGURE 4.** HFD-induced obesity alters lung metabolic pathways during pH1N1 infection. (A-C) OPLS-DA plot and differentially altered metabolites of lung samples from uninfected (A), 4 dpi (B), and 8 dpi (C) CD and HFD mice (\(n = 5–6\)). Next to each metabolite, \(\ast p < 0.05\), \(\ast \ast p < 0.005\), and \(\ast \ast \ast p < 0.0005\), and numbers in parentheses represent the number of species for that metabolite that are significantly different between HFD and LFD (i.e., methodology was not able to differentiate the multiple species). (D) Pathway enrichment analysis generated in GeneGo of metabolic pathways significantly different between CD and HFD mice at 8 dpi. ChREBP, carbohydrate-responsive element–binding protein; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid.
mice displayed a lower percentage of body weight lost compared with lean LepRHfl/fl mice. (A and B) Weight gain of male (A) and female (B) mice lacking leptin receptor signaling in hypothalamic neurons LepRH2/2, heterozygous mice LepRH+/2, and fully floxed LepRHfl/fl mice (n ≥ 9). (C and D) Mortality curves for male (C) and female (D) mice postinfection with 5.8 × 10^7 TCID$_{50}$ pH1N1 at 14–16 wk of age (n ≥ 9). (E and F) Total weight loss for male (E) and female (F) mice after pH1N1 infection (n ≥ 9). (G and H) Percentage weight loss for male (G) and female (H) mice after pH1N1 infection (n ≥ 9). Each datum point represents mean ± SEM. In (A)–(F), *p < 0.05, **p < 0.005, or ***p < 0.0005 comparing LepRH2/2 with both LepRH+/2 and LepRHfl/fl mice; for (G) and (H), **p < 0.05 or ***p < 0.0005 comparing LepRH2/2 with LepRHfl/fl mice and *p < 0.05 or **p < 0.0005 comparing LepRH+/2 with LepRHfl/fl mice.

Obese LepRH2/2 mice exhibit greater lung inflammatory responses and alterations in lung immune cell populations during pH1N1 infection

Given the elevated mortality in the LepRH2/2 obese mice compared with lean controls, we next assessed pathological and immunological responses in the lungs of LepRH2/2 mice. Fig. 6A demonstrates that whereas LepRH2/2 mice had significantly larger mesenteric white adipose tissue depots and livers compared with lean LepRHfl/fl mice, there were no differences in lung weight. Lung viral titers were then measured, and LepRH2/2 did not exhibit differences in viral burden (Fig. 6B). However, the obese LepRH2/2 mice did have fewer infiltrating cells into the airways during the infection (Fig. 6C), but they exhibited a greater level of injury to the lung epithelium compared with lean LepRHfl/fl mice at 8 dpi (Fig. 6D). Gene expression of lung cytokines and chemokines also demonstrated that the genetically obese mice had greater pulmonary inflammation at 8 dpi (Fig. 6E). LepRH2/2 mice exhibited higher levels of IL-10, MCP-1, and KC at 8 dpi.

In the dietary model of obesity, HFD mice had fewer BAL macrophages and Tregs. Therefore, the distribution of these cells in LepRHfl/fl and LepRH2/2 mice was assessed at 4 and 8 dpi (Fig. 6F–H). Similar to HFD obese mice, LepRH2/2 mice had fewer BAL macrophages (Fig. 6F). Additionally, LepRH2/2 mice also had fewer CD4+ T cells, CD8+ T cells (data not shown), BAL Tregs, and activated BAL Tregs (Fig. 6G, 6H). Therefore, both HFD and LepRH2/2 obese mice exhibit similar differences in BAL macrophage and Treg numbers during pH1N1 infection in comparison with lean controls.

Obese LepRH2/2 mice exhibit distinct metabolic perturbations in lung tissue during pH1N1 infection

To enhance our analysis of the metabolic consequences of obesity during pH1N1 infection, the lungs of LepRHfl/fl and LepRH2/2 mice were harvested at 0 and 8 dpi and processed for metabolic profiling. In Fig. 7A and 7B, the OPLS-DA plots demonstrate...
Of interest, most metabolites different between LepRH^{fl/fl} and LepRH^{+/+} during pH1N1 infection, rather than being caused by obesity alone. Metabolic data at 8 dpi are specific to the consequences of obesity. 0 dpi were also different at 8 dpi, indicating that the recovered alterations in a number of lipid metabolites at both 0 and 8 dpi. Additionally, few metabolites differentially affected by obesity at 8 dpi. This time point occurs prior to drastic weight loss or obvious signs of sickness, allowing utilization of metabolic profiling for identifying biomarkers that may be used to predict or explain mechanisms of greater severity in LepRH^{+/+} obese mice. The OPLS-DA plot in Fig. 8A demonstrates that UPLC-MS profiling was able to successfully distinguish urine from LepRH^{+/+} and LepRH^{+/−} mice at 0 and 2 dpi. A relatively large number of metabolites were detected at greater levels in the urine at 0 dpi in LepRH^{+/+} mice such as L-acetylcarnitine, phosphatidylcholine, and eight different amino acid–related metabolites (Fig. 8C). Lastly, pathway analysis of urine metabolomes at 2 dpi revealed alterations in pathways related to fatty acid, amino acid, and histamine metabolism (Fig. 8D). Although it is known that obesity is characterized by alterations in fatty acid metabolism, we show that changes in fatty acid metabolic pathways (and several other pathways) occur systemically in the urine and locally in the lung during a pH1N1 infection.

**Discussion**

Obesity is a global epidemic, and the consequences of excess adiposity are diverse and continue to mount (43). Following the emergence of the 2009 pH1N1 strain, obesity was reported to be an independent risk factor for greater pH1N1 (4, 6, 17, 37, 38) and seasonal (44–46) influenza infection severity. In this study, we provide an in-depth analysis of the immunologic and metabolic complications associated with obesity during infection with the 2009 pH1N1 influenza. Previous studies in mice have shown that obesity alters lung wound healing and inflammatory responses during a pH1N1 infection (10), possibly due to hyperleptinemia (11). However, the complications of obesity are complex, and a number...
of other factors could be affecting pH1N1 immunity such as hyperglycemia, hyperinsulinemia, oxidative stress, changes in the gut microbiome, and alterations in cellular and systemic metabolism (9). Therefore, we sought to combine immunological assessments with metabolomics to provide a more global characterization of the metabolic consequences of obesity on pH1N1 infection.

One of several mechanisms in which HFD-induced obesity may result in greater pH1N1 severity is the impact of consuming a HFD. Altering dietary composition, even without inducing nutritional deficiencies, can have systemic metabolic consequences (47–49) and can affect antiviral immunity (50). One inherent limitation with a diet-induced obesity model is that a HFD will always differ from a lean control diet (e.g., saturated fatty acid content, carbohydrate content), potentially confounding the implications of the impact of obesity. One goal of this investigation was to provide an in-depth analysis of the consequences of HFD-induced obesity in comparison with two different lean control groups to better assess dietary contributions. It has previously been demonstrated that LFDs high in sucrose can have metabolic consequences and can impact immune responses (51). Additionally, the CD differs in nearly every aspect from the purified LFDs and HFDs (yet it is still one of the most widely used control diets for obesity studies) (52). Several key differing components are amounts of phytoestrogens and dietary fiber. CDs are primarily derived from plant constituents and therefore contain phytoestrogens as well as dietary fiber (52). Phytoestrogens have been shown to affect metabolism and behavior. Furthermore, fiber can also impact metabolic health and modulate the gut microbiome (52, 53), and the purified LFD has no fiber (perhaps contributing to greater weight gain and a somewhat intermediate metabolic phenotype between CD mice and HFD mice as in Fig. 1). These differing characteristics of the diets likely explain some of the differential responses observed...
Currently, three studies have demonstrated that diet-induced obese mice exhibit greater mortality to a primary pH1N1 infection (10, 11, 15). Two of these studies used a CD for the lean control group and a 45 or 60% kcal HFD for obese mice (10, 11), and one study used a 60% kcal HFD compared with a nutrient-matched, purified 10% kcal LFD (15). Although we found that HFD mice were more susceptible compared with both lean groups, lean LFD mice exhibited greater mortality compared with lean CD mice. This demonstrates that modulating the diet alone, without inducing obesity, can increase pH1N1 severity, and it is possible there are synergistic effects of being obese and consuming a HFD. This outcome may have potential public health implications in that perhaps not only being obese increases risk for influenza severity, but the composition of the diet one chooses to eat could also impact severity as well.

Given the complications of diet-induced obesity models, a genetic model of obesity was also included in our analysis in an attempt to better assess the immunological consequences of excess adiposity independent of dietary effects. Obese male and female LepRH\(^2\)/\(^2\) mice were significantly more susceptible to pH1N1 mortality compared with lean control groups.

**FIGURE 8.** Metabolic profiling reveals alterations in distinct metabolic networks in urine of pH1N1-infected obese LepRH\(^{+/−}\) mice. (A) OPLS-DA plot from urine samples from the same cohort of uninfected and 2 dpi mice. (B and C) Differentially altered metabolites in the urine of uninfected (B) and at 2 dpi (C) LepRH\(^{+/−}\) mice compared with lean LepRH\(^{R+/R} \) mice (\(n = 3\)). (D) Pathway analysis generated in GeneGo of all pathways significantly different at 2 dpi between LepRH\(^{R+/R}\) and LepRH\(^{+/−}\) mice. Next to each metabolite, \( ^* p < 0.05 \), \( ^{**} p < 0.005 \), or \( ^{***} p < 0.0005 \). #, 1-nitro-7-glutathionyl-8-hydroxy-7,8-dihydronaphthalene; PPAR\(γ\), peroxisome. proliferator-activated receptor \(γ\).
leptin are a commonly used model of genetically induced obesity, in which lack of leptin satiety cues result in hyperphagia and obesity (54, 55). O’Brien et al. (10) have previously demonstrated that obese mice were more susceptible to pH1N1 mortality. Although this is informative and helps to address the complications of dietary models discussed above, a global deficiency of leptin signaling can cause physiologic and immunologic complications, confounding the effects of obesity (55). Therefore, we used a genetic model of obesity in which disruption of leptin signaling is isolated to hypothalamic neurons (although there is some evidence of Nkx2.1 expression in the lungs, esophagus, and during development) (32). This genetic model of obesity established that although diet may synergize with obesity to impact pH1N1 immunity, obesity alone is sufficient to increase pH1N1 mortality. Additionally, this study highlights the importance of careful consideration in choosing a mouse model of obesity (genetic- versus diet-induced) and in selecting proper dietary controls for HFD-induced obesity studies.

Both diet- and genetic-induced obese mice exhibited greater lung damage during the pH1N1 infection. This excess lung pathology has been demonstrated previously in obese mice, and it is likely due to impaired wound healing in the lung (10). A number of potential mechanisms may be responsible for enhanced lung damage in obese mice. Tregs are critical regulators of immunopathology and have been shown to limit inflammatory responses to respiratory syncytial virus and some influenza infection models in mice (39–41). We have previously demonstrated that obese mice have fewer Tregs in the lung airways during a secondary pH1N1 challenge, and Tregs isolated from obese mice exhibited impaired suppressive capacity compared with Tregs isolated from lean mice (14). Therefore, we also investigated the distribution of Tregs in the lung, lung airways, and mLN during a primary pH1N1 infection. At 8 dpi, HFD- and genetic-induced obese mice had fewer BAL Tregs and fewer activated BAL Tregs. It is currently unclear why obese mice have fewer BAL Tregs during influenza infection, but it is well established that obesity results in a deficiency of Tregs in metabolic tissues such as the liver and white adipose tissue (8, 9). Obesity results in greater lung damage during both primary and secondary pH1N1 infections, and therefore uncovering how obesity modulates Treg responses is likely important for understanding the complex mechanisms of influenza severity caused by an obese state.

Additionally, fewer F4/80+ macrophages were detected in the lung airways of obese mice at 4 dpi in HFD mice and at 8 dpi in LepRH−/− mice. NK cell numbers were also slightly lower in HFD mice than in CD mice, whereas most metabolites in LepRH mice differ greatly highlights the impact of the diet in this model. Future studies are required to better understand the synergistic effects of a HFD and excess adiposity, as well as how these factors interact to modulate the lung metabolome and affect pH1N1 immunity.
We also performed metabolic profiling on urine samples from LeprH<sup>+/−</sup> and LeprH<sup>−/−</sup> mice at 2 dpi. Similar to changes in lung metabolites, alterations in urinary metabolites related to fatty acid, nucleotide, and amino acid metabolism were measured. The fact that metabolic profiling can distinguish Lepr<sup>−/−</sup> mice from lean control mice as early as 2 dpi (prior to any obvious signs of illness or drastic weight loss) introduces the possibility of utilizing metabolic profiling to complement current prognostic or diagnostic approaches in the future. Additionally, the urinary metabolome may provide insight into the mechanisms in which obesity increases pH1N1 mortality. For example, taurine was elevated in the urine of obese LeprH<sup>−/−</sup> mice at 2 dpi. Detection of taurine has been reported to be increased under conditions of oxidative stress (63). The fold change of taurine increased from slightly greater in uninfected urine of LeprH<sup>−/−</sup> mice to 4-fold greater by 2 dpi. Therefore, this may indicate that influenza infection induces greater oxidative stress in obese mice.

Taken together, we have provided a comprehensive analysis of both the immunologic and metabolic consequences of obesity during pH1N1 infection. It is likely that the distinct metabolic perturbations detected in the lungs of obese mice are both reflective and infillactic of pathologic changes occurring in the lung following infection. Additionally, we were able to document distinct changes in the urine of infected mice before overt signs of illness, demonstrating the strength of this approach. Future studies are required to further integrate these metabolomic data with immunologic responses to identify novel mechanisms for greater pH1N1 severity in obese mice.

Disclosures

The authors have no financial conflicts of interest.

References


